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Exploration of potential mechanisms of HCV resistance in exposed uninfected intravenous drug users

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TITLE PAGE

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Abstract

A rare outcome following exposure to hepatitis C virus (HCV) is a lack of observable infection as clinically measured by HCV RNA or HCV recognising antibodies. The population who exhibit this trait is termed exposed uninfected (EU). Increasing evidence has refined characterisation of these individuals, distinct from those who become infected but spontaneously clear HCV. Study of the EU population is highly pertinent for the discovery of antiviral mechanisms of resistance that can reveal antiviral therapeutic strategies.

This review provides an overview of similarities and differences of the EU population relative to spontaneous resolvers and the majority whom develop chronic HCV infection, and focusses on possible mechanisms of resistance including innate and adaptive immunity, genetics and lipid interactions.

Key words:

Cytokines; Exposed uninfected (EU); Hepatitis C virus (HCV); Immune response; Liver; people who inject drugs (PWID); Resistance

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Introduction

Hepatitis C virus (HCV) infection is (1) a major global public health problem that can result in cirrhosis, liver cancer and death with estimates of prevalence of antibodies against HCV (anti-HCV) ranging between 1.3 – 2.1 % (2). Injection drug use is the dominant risk factor for HCV transmission in economically developed countries. Longer duration of injecting drug use is associated with increased prevalence of HCV infection (64 – 94% HCV anti-HCV positive) (3,4). Epidemiological data indicates that globally 60 – 80% of people who inject drugs (PWIDs) are anti-HCV positive, with over 80% prevalence in 12 countries (5). This equates to approximately 10 million PWIDs who are anti-HCV positive worldwide, with an estimated 1.6 million in China, 1.5 million in the United States of America, and 1.3 million in Russia (5).

There is evidence that a small proportion of PWIDs remain HCV RNA and anti-HCV negative despite their history of repeated exposure to HCV through sharing of injecting needles and other paraphernalia. Our group and others have previously characterised this cohort of PWIDs defining them as 'exposed uninfected' (EU) (6–10). The phenotype of repeated exposure to HCV without the development of infection is of considerable interest because it suggests these individuals are in some way resistant to HCV infection. This review considers the phenotypic definitions of the EU cohort, and the distinct immunological and genetic features that characterise the EU group to gain insights into possible mechanisms of HCV resistance.

Differences between HCV Exposed Uninfected (EU), Spontaneous Resolvers (SR) and those with chronic HCV (CHCV)

Parenteral HCV exposure results in one of 3 different outcomes: the majority of individuals develop chronic HCV infection (CHCV) with detectable anti-HCV and persisting HCV RNA, while approximately 20% of people spontaneously resolve infection (SR) (11) with detectable anti-HCV but clearance of HCV RNA, and finally a minority, who despite similar viral exposure, remain uninfected (EU) lacking detectable anti-HCV and HCV RNA. These 3 outcomes can be characterised by differing immunological and genetic profiles (Table 1).

HCV 'exposed uninfected' designation

HCV exposure without infection has been described by several groups. While we have termed this cohort 'exposed uninfected' (6), other groups describe a similar phenomenon in both HCV and Human immunodeficiency virus (HIV) as 'highly exposed seronegative (HESN)' (12), 'highly exposed persistently seronegative (13), repeatedly 'exposed seronegative (ESN)' (14). For meaningful debate and understanding, there needs to be clarity about phenotypic nomenclature.

Exposed uninfected individuals represent a distinct group

In the absence of serological evidence for developing infection, HCV exposure can only be determined by probability based on the history of risk behaviour. It is known that PWIDs who share needles or other injecting paraphernalia with individuals known to have HCV infection are at high risk of HCV exposure and that HCV sero-prevalence increases with duration of injection history (15,16) as the cumulative probability of actual exposure increases.

The EU group therefore needs to be carefully distinguished from *unexposed* naïve cases by close scrutiny of the probability of actual HCV exposure. EU groups are practically defined as individuals whose test results are serially negative for anti-HCV and HCV RNA on at least 2 occasions, more than

6 months apart, in the context of ongoing high risk of HCV exposure, typically through injection drug use and sharing of injection paraphernalia (7,17). The probability of true HCV exposure has mostly been determined on the basis of risk questionnaires. An example utilising exceptional high-risk behaviours was described by Sugden et al, using a composite risk index for HCV exposure based on a time dependent cox-regression analysis of 14 separate weighted risk factors of risk behaviours and demographics (18). Significantly higher composite risk scores were found in those that subsequently became infected. However, within the uninfected group there were individuals that remained uninfected despite high risk behavioural profiles. Separation of the uninfected group's risk into tertiles (two points that divide data into three equal parts) indicated that those in the highest tertile had a pattern of risk actually higher than that observed in subjects who subsequently become infected (18). Thus based on statistical models it is highly probable that these subjects had been exposed to HCV but despite this remained uninfected.

An alternative definition of HCV exposure in EUs is the presence of HCV specific immunological responses (18) in the absence of anti-HCV or HCV RNA. T cell responses can be measured to arrays of HCV peptides spanning structural and non-structural (NS) viral proteins, and are a marker of immunological exposure to HCV. ELISpot is a technique employed for detection and analysis of individual cells that secrete cytokines in response to in-vitro stimulation with specific antigens (in this case HCV peptides)(19) and is highly sensitive allowing for detection of as few as one cell in 100,000 (20). Cellular IFN-γ production is used as a readout of single cell activation with individual spots representing each cytokine secreting cell (20). Using ELISpot up to 60% of high risk individuals have detectable HCV-specific T cell responses (6).

Both definitions of HCV exposure have their flaws, with risk stratification according to injection history dependent on self-reported behaviours, and immunological definition potentially excluding cases who have alternative pathways of resistance and who don't mount a T cell response despite viral exposure. Despite these limitations it is clear that in some individuals repeated parenteral

exposure to HCV through drug use does not result in infection becoming established and defining mechanisms of resistance in these cases is a key question for HCV research.

Immunological resistance to HCV

Given the high prevalence of T cell responses in the EU cohort, immunological mechanisms may be an important component of HCV resistance and the data suggesting differences in adaptive and innate immune responsiveness in EU compared to others is reviewed here.

a) adaptive immune responses

i. T Cells

Spontaneous resolution of HCV infection is associated with robust T cell responses (21,22) and early, vigorous and multi-specific T cell responses targeting multiple viral epitopes appear crucial for spontaneous clearance (reviewed in (23)). Conversely, an ineffective early cell-mediated response may result in the development of chronic HCV infection (24). In SRs viral clearance is dependent on a complex set of viral-host interactions such as young age (25); females respond better than males (26), host immune response and genetics, neutralising antibodies, and viral specific factors that include genotypes, and diversity of quasispecies (27).

Partial protective immunity after spontaneous resolution of HCV infection has been reported, with lower rates of HCV reinfection than would be expected in a matched uninfected population (28). Detectable HCV-specific T cell responses remain for many years after spontaneous clearance (29) but their role in providing this partial protection has not been established.

In exposed uninfected cases up to 60% have demonstrable T cell responses (6), often to multiple viral antigens, but these responses are typically much weaker than those seen in SR (7,8,17). Furthermore these T cell responses wane rapidly on cessation of injection drug use, and seem to require continued HCV exposure as a consequence of ongoing injection drug use to be maintained (30). HCV specific cytotoxic T cell responses have been reported in imprisoned PWIDs in association with transient hepatitis C viraemia that cleared without seroconversion (reviewed in (31)). Similar HCV-specific T cell immune responses have been described in avireamic and seronegative family members of HCV infected individuals (32,33) and in healthcare workers following needle stick exposure to HCV with evidence of an early T cell response induced in the absence of detectable anti-HCV or HCV RNA (34). Overall these studies suggest that low dose exposure to HCV can prime HCV specific T cells and that repeated exposure can maintain that response (32,33,35) so that the frequency and dose of HCV exposure may influence any resulting degree of protection.

The degree to which T cell responses confer protection in EUs is controversial. A nested case control series from a prospectively studied cohort of PWIDs in Australia revealed no differences in T cell responses assessed by HCV-specific IFN-γ ELISpot, and HCV-specific CD4 T effector responses between those who developed incident HCV infection compared to those who remained uninfected (EU) suggesting that whilst HCV-specific cellular immunity is prevalent in EU subjects it did not correlate with protection in their cohort (36).

In keeping with this lack of correlation between demonstrable T cell responses and protection from HCV infection, other studies have shown clearance of low levels of HCV viraemia without developing anti-HCV antibodies can occur in the absence of any demonstrable T cell immune responses (37,38).

In conclusion, whilst HCV-specific T cell immunological responses do provide good evidence of viral exposure, their role in conferring any protection from infection remains unproven, which suggests that there are other mechanisms involved in resistance to HCV infection.

ii. B cells and anti-HCV mediated protection

There is growing evidence that a robust anti-envelope antibody response can contribute significantly to viral clearance in acute infection (39) with the rapid onset of anti-HCV capable of neutralizing diverse strains of HCV being associated with clearance (40). Furthermore, in vitro and in vivo studies have suggested that broadly neutralizing antibodies may contribute to resolution of infection even once HCV has become established (41,42). Studies in chimpanzees utilising HCV neutralizing antibodies have indicated that viral replication can be delayed after challenging chimpanzees with pre-existing anti-HCV (43). Robust neutralising antibody responses not identified by conventional HCV antibody assays may therefore contribute to HCV resistance in EUs. We recently reported on finding significant IgG reactivity to HCV envelope proteins in 20 of 42 HCV antibody and HCV RNA negative EU cases, with 6 of them showing evidence of a significant virus neutralizing effect (44). IgG from the neutralizing samples did not compete for binding sites with several well-characterized antibodies to known conformational epitopes on the E2 glycoprotein, suggesting novel epitopes may be targeted (44). This is the only report to date of HCV-specific humoral immune responses in exposed uninfected injection drug users. Current work seeks to identify if humoral responses correlate with the presence of T cell responses. The presence of specific anti-envelope antibodies provides further confirmation of HCV exposure in these cases and is a plausible, but as yet unproven, mechanism by which some of these subjects may have resisted HCV infection following a low dose inoculation.

b) Innate immunity

The innate immune system is the first line of defence against viral illness with the potential to prevent infection as well as facilitate subsequent adaptive immune responses and modify outcome of established infection. Natural killer (NK) cells are considered the principal innate effectors and when activated can kill either directly via the release of granzyme and perforin containing cytotoxic granules or via cytokines such as IFN-γ. NK cells also act indirectly by influencing the activation and/or trafficking of other key immune cell populations, including T cells, to promote adaptive immune responses.

Natural Killer cells are highly enriched in the liver in comparison to peripheral blood (45). NK cell activation is tightly controlled through a complex balance of activating and inhibitory receptors, with inhibitory NK receptors (NKRs) in steady-state conditions dominating signals from activating receptors, to safeguard from NK cell reactivity toward normal, healthy cells. The main classes of NKRs are the predominantly inhibitory killer immunoglobulin-like receptors (KIR), the natural killer group 2 (NKG2) family of inhibitory (NKG2A) and activatory (NKG2C/D) isoforms, and the activating natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46.

There is increasing evidence to support a key role for innate immune responses in EU cases. A study looking at serum cytokine profiles in EU cases found significantly elevated levels of the pro-inflammatory innate immune cytokine interleukin-6 and chemokine interleukin-8 in EU's compared to those with chronic HCV, spontaneous resolvers or healthy controls (46). A genetic study of the NK cell KIR receptors identified homozygosity for KIR2DL3:HLA-C1, which is linked to a readily activated NK cell phenotype (47), to be associated with the resistance to HCV infection seen in EU cases (48). Of interest, the IL28B variant strongly associated with spontaneous resolution of HCV infection and a favourable treatment outcome was not found to be associated with these EU cases indicating that EU individuals are a distinct population from patients who spontaneously resolve HCV infection (48).

More recently studies have reported phenotypic and functional differences in NK cells in high-risk cohorts that do not develop HCV infection. In the first HCV seronegative PWIDs were shown to have increased levels of KIR2DL3^{+}NKG2A^{-} NK cells compared to PWIDs with chronic or resolved HCV infection and these cells were not susceptible to HLA-E mediated inhibition (49). The strongest evidence to date comes from a prospectively studied cohort of seronegative PWIDs in Australia (36). This nested case-control series studied 28 previously seronegative cases who seroconverted (incident cases of HCV) matched by demographics and risk behaviour to 28 EU subjects who remained seronegative on follow up. Samples were assayed for NK cell phenotypes and function before and after incident HCV infection to seek correlates with ongoing resistance to HCV in those who remained uninfected. Sustained NK cell activation was found to contribute to protection against HCV infection with significantly higher numbers of both activated and cytotoxic cells found in the EU subjects together with higher frequencies of interferon gamma (IFN-γ) producing NK cells. In contrast, no correlation with HCV-specific T cell responses and ongoing protection was identified.

c) Other possible mechanisms of HCV resistance

i. Genetic

To date genetic studies in EU cases have been limited by the relatively small cohorts identified and reported on, so that studies have focussed on specific candidate genes. Further collaborative investigations combining different EU cohorts will be needed to undertake any appropriately powered genome wide association study. Candidate genes include those that influence the host innate/and or adaptive immune responses as well as those encoding HCV receptor molecules.

We have previously shown an association between the variant IL-12B C allele and resistance to HCV infection in EU cases (50). This variant is associated with higher IL-12 production (51) and thus may be of relevance in promoting effective anti-viral immune responses. As described in the section on innate immune responses, a study on the genetics of NK cell KIR receptors identified a variant associated with a readily activated NK cell phenotype (KIR2DL3:C1 homozygotes) (47), to be associated with the resistance to HCV infection seen in EU cases (48).

HCV entry into hepatocytes is a complex multi-step process that involves a number of host factors and co-receptors including CD81, Scavenger receptor class B member 1 (SRB1), the tight junction proteins claudin 1, 6 and 9 and occludin as well as epidermal growth factor receptor (EGFR), Ephrin receptor A2 (EphA2), transferrin receptor 1 (TfR1), Niemann-Pick C1-like 1 (NPC1L1) and others (reviewed in (52)).

CD81 appears to be very highly conserved with no genetic alterations found in a study of EU cases, HCV infected cases and controls (53). Studies reported on a French cohort of HIV infected PWIDs with and without HCV infection (the HCV negative cases were considered EU) looked at sequencing findings in 22 EU cases and found no alteration in Claudin-1 (54), but did find heterozygous variants in claudin-6 and occludin in one case and two heterozygous variants in SRB-1 in another (55). The relevance of these to resistance to HCV is unclear as detailed study showed that the variations in Claudin-6 and occludin identified did not confer resistance to HCV infection in an in-vitro model (56).

ii) **Lipids and HCV resistance**

HCV interacts with host lipid metabolism at all stages of the viral lifecycle - from attachment and entry into hepatocytes, to replication and assembly of new viral particles. Circulating HCV is

associated with lipoproteins as complex 'lipoviral particles' (LVP) (57) which contain viral RNA, viral proteins, and host apolipoprotein constituents including apoB, apoE, apoA1, and apoC (58). Growing evidence suggests that the LVP lipid and apolipoprotein components facilitate viral attachment to host cells by binding to cellular lipoprotein receptors and that the exchangeable apoE appears to be crucial for infectivity at the attachment step and at masking envelope glycoproteins from neutralising antibodies (59). HCV replication takes place on lipid droplets with co-localization of core protein and non-structural NS5A to the lipid droplet. Production of infectious HCV is dependent on hepatocyte machinery for the export of very low density lipoproteins (VLDL) (60).

Thus evidence points toward a key role for lipid metabolism in the HCV lifecycle, and the close association with lipids and lipoproteins contributes to HCVs ability to evade the host's immune surveillance. Given this co-dependency on host lipid pathways, it is plausible that any mechanism that would disrupt the LVP formation would potentially reduce the viral infectivity and influence outcome following HCV exposure.

Recently reported, but preliminary, data using lipidomics profiling demonstrated that the HCV resistant EU phenotype was clearly distinct from HCV susceptible individuals (61). The role of lipids and lipid pathways in conferring HCV resistance is of interest but remains to be clarified.

Conclusion

HCV exposure through injection drug use that does not result in infection is now well described in several countries. This EU group exhibit resistance to HCV infection. Detailed study to date suggests that this resistance is associated with an increased likelihood of having adaptive HCV-specific T and B cell responses, and an upregulated innate immune response. Although the role these HCV-specific responses in conferring resistance to HCV infection remains ambiguous, detection of these responses confirms exposure in the absence of anti-HCV immunoglobulin or HCV RNA. Possible mechanisms of resistance include upregulated innate immune responses including cytokine production, genetic variation, and altered lipid composition. Current evidence suggests that the EU phenotype represents a spectrum, with varying degrees of resistance to HCV, possibly involving complex interactions between the inoculation size and frequency of HCV exposure with immune mediated pathways and other host factors such as genetic polymorphisms.

The accurate characterisation of HCV exposed uninfected cases remains crucial to future studies and raises issues regarding the definition and confirmation of HCV exposure. There remains a need for a universally agreed definition of exposure on the basis of probability derived from risk behaviours and duration of drug use, and / or evidence of immune responsiveness to HCV antigens, in order to define robust exposed uninfected cohorts to investigate pathways of HCV resistance. A collaborative effort between groups using agreed definitions for EU cases could provide sufficient case numbers to undertake appropriately powered genetic studies. A better understanding of all the factors associated with HCV resistance would be a major step forward in our understanding of the pathogenesis of this highly prevalent infection and in the search for alternative therapeutic strategies.

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Tables

Immunological and genetic differences based on HCV natural history

	Exposed Uninfected	**Spontaneous resolution**	**Chronic Infection**
Anti- HCV	Persistently negative	positive	positive
HCV RNA	Persistently not detected	Transient positive, then persistently negative	Two positive > 6 months apart
Neutralising antibody responses	Detected in 27% (62)	Associated with early development of broadly neutralising antibody responses (40). And anti-host receptor (CD81) antibodies (63).	Readily detectable neutralising antibodies in chronic infection
T cell responses	Detected in 60% but weak and wane on cessation of HCV exposure (17)	Early vigorous and broad anti HCV T cell responses typical (23).	Initial T-cell responses weaken. Loss of CD4 T-cell help, switch to a T-reg cell profile, viral epitope escape, chronic antigen stimulation contribute to T-cell exhaustion (64).
Genetic factors	↑ KIR2DL3 HLA-C (17). Not IFNλ3 (48)	↑ KIR2DL3 HLA-C (17) IFLλ3 CC (48)	↓ KIR2DL3 HLA-C (17). IFNλ3 (48)